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Non-Isotopic Competitive Reverse Transcription Polymerase Chain Reaction Coupled with High Performance Liquid Chromatography to Measure β_2-Receptor Messenger RNA in the Human Heart

Klaus Höhnel[1], *Dieter Ratge*[1], *Jeanette Giray*[1], *Christiane Lauk*[1], *Theresia Rose*[2], *Klaus Hellberg*[3] and *Hermann Wisser*[1]

[1] Department of Clinical Pathology,
[2] Department of Anaesthesiology,
[3] Department of Cardiovascular Surgery,
Robert-Bosch-Krankenhaus, Stuttgart, Germany

Dedicated to Prof. Dr. Dr. J. Büttner on the occasion of his 65th birthday

Summary: We describe an application of competitive reverse transcription-polymerase chain reaction (PCR) coupled with HPLC for quantification of β_2-adrenergic receptor messenger RNA (mRNA) in human atrial tissues removed during cannulation for cardiopulmonary bypass operations. We constructed an internal standard which was reverse transcribed in different concentrations together with constant levels of cellular RNA and subsequently PCR amplified. The competitor RNA shows the same β_2-adrenergic receptor primer sequences as the cellular mRNA but yields a different-sized product. This allows resolution of the amplified copy DNA (complementary DNA, cDNA) fragments with a specific HPLC column. The concentration of β_2-adrenergic receptor mRNA is derived from the ratio between the peak intensities corresponding to the amplified competitor and target products. We assessed the imprecision, accuracy and sensitivity of the method. Concentrations of β_2-adrenergic receptor mRNA of $22.7 \pm 15.2 \times 10^6$ molecules per µg total RNA in patients treated with β_2-antagonists were not significantly different from control patients showing $16.8 \pm 9.9 \times 10^6$ β_2-adrenergic receptor mRNA molecules per µg total RNA (Mean ± SD). Competitive reverse transcription PCR is a highly specific, non-radioactive procedure for quantification of β_2-adrenergic receptor mRNA and simultaneously other gene expression levels of interest in atrial tissue specimens and may therefore be used to advance our understanding of heart muscle disease.

Introduction

The β-adrenoceptor antagonists are widely used in the treatment of angina and hypertension. Abrupt cessation of β-blocker treatment may be associated with adverse clinical events, such as the β-blocker withdrawal syndrome (1). Treatment with β_1-selective antagonists leads to increased cardiac β_1-adrenoceptor density with no change in β_2-adrenoceptor density (2). On the other hands, the cardiac β_2-adrenoceptor responsiveness was markedly increased due to enhanced coupling of the β_2-adrenoceptor to effector mechanisms after atenolol treatment (3). The subject of the present study was to determine whether the unchanged β_2-adrenoceptor density with increased β_2-adrenoceptor responsiveness after β_1-blocker treatment is linked with alterations in gene expression.

Although measurements of messenger RNA (mRNA) would be useful in advancing our understanding in human heart disease, determinations of mRNA in human tissues have been limited to the large amount of myocardium that can be obtained from patients who are undergoing cardiac transplantation (4). Quantitative reverse transcription polymerase chain reaction (PCR) is being increasingly used as an alternative to detect and quantify gene expression from minute amounts of RNA (5). However, variation in gene expression can only be compared between different samples if a common factor relates the samples to be compared. Moreover, the efficiency of the RNA extraction and reverse transcription steps needed before PCR amplification cannot be accurately monitored. Nevertheless, quantification of an mRNA species by reverse transcription PCR amplification can be achieved by measuring the amount of a PCR product obtained from a target gene mRNA sequence (in this study, β_2-mRNA) relative to the amount of the PCR product coamplified from known amounts of added competitor mRNA (internal standard). As the PCR products only differ in their length, it can be assumed that the reverse transcription PCR efficiencies are essentially equal for β_2-mRNA and the internal standard. In this procedure, the *PCR aided transcript titration assay*

(PATTY) described by *Becker-André & Hahlbrock* (6), identical portions of total cellular RNA are spiked with increasing amounts of internal standard RNA. After reverse transcription, the resulting copy DNAs (cDNAs) are amplified in a PCR assay where a competitive primer annealing reaction between internal standard and target sequence takes place. Within the linear range of amplification, the doubling of the competitor cDNA corresponds to an approximate doubling of the target cDNA for a given number of cycles.

Current techniques for detection and accurate quantification of the small DNA fragments may be time-consuming and hazardous. These procedures often use radiolabelled nucleotides or primers and gel electrophoresis in the presence of ethidium bromide (7, 8). Here we present an alternative purification protocol using a computerized HPLC device with ion exchange chromatography (9) to separate each PCR product from free nucleotides and excess primer. We also optimized the assay conditions and determined the reliability of this reverse transcription PCR/HPLC protocol to readily quantify β_2-mRNA expression in minute clinical samples.

Materials and Methods

Subjects and tissue preparation

Two groups of 10 patients undergoing elective cardiac cardiopulmonary bypass were recruited. All patients were clinically or chemically euthyroid and none had severe ischemia. We selected patients suffering from cardiac artery disease with heart failure (New York Heart Association class) II–III and III.

One group of patients had been treated with β_1-antagonists for at least 3 months (metoprolol 100 mg/d (n = 6), bisoprolol 5 mg/d (n = 3), atenolol 50 mg/d (n = 1). The other group had not taken β-blockers for at least 3 months. None of the patients had received calcium-antagonists, angiotensin converting enzyme inhibitors or catecholamines.

In both groups, additional medication consisted of the cardiac glycoside digoxin (given to approximately half of the subjects) and nitrates (given to approximately half of the subjects). Patients received the medication up to the morning of surgery.

The cardiac tissues (right heart auricle) were removed during cannulation for cardiopulmonary bypass operations. Patients were normothermic at the time the tissue was removed. The heart tissues were immediately frozen in liquid nitrogen and stored at −70 °C until further processing.

Extraction of RNA

Total RNA was isolated by fractionated precipitation and anionic exchange chromatography according to the manufacturer's protocol ("RNA-Isolationskit", Qiagen, Hilden, Germany; (10)). RNA preparations were free from traces of remaining genomic DNA, indicated by PCR without the reverse transcription step obtaining no signal (negative control). The concentration of isolated RNA was determined at 260 nm. The 260 nm : 280 nm absorbance ratio of the solution was found to be 1.8 to 2.0 which reveals high purity of the RNA. Yields of RNA extraction varied between 8–17%.

Preparation of internal standard RNA

In reverse transcription PCR experiments for quantification of specific mRNA-species, one requires an internal standard RNA as competitor. Therefore, we constructed a plasmid containing specific primer annealing sites identical to the target annealing sites. In vitro transcription of this sequence provides the competitor RNA for quantitative reverse transcription PCR. For construction, we purchased two single-stranded DNA-sequences which were complementary in 29 nucleotides at their 3'-ends (fig. 1). They possess specific primer annealing sites for PCR-amplification of atrial na-

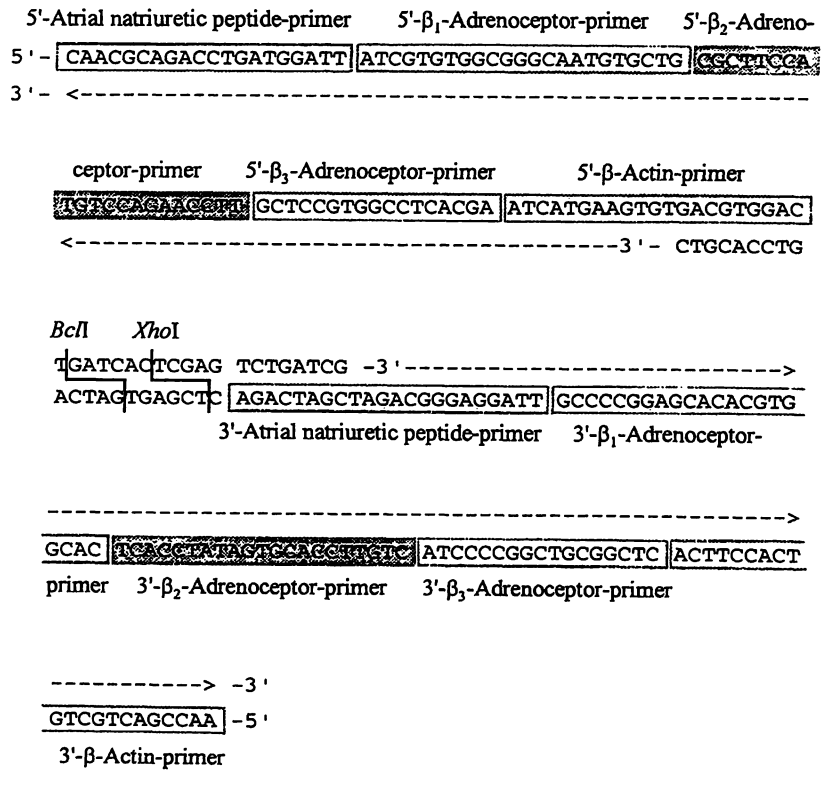

Fig. 1 Construction of the internal standard DNA.
Two single stranded DNA-sequences, complementary in 29 bases at their 3'-end were PCR-amplified by use of the 5'-atrial natriuretic peptide primer and the 3'-β-actin primer as described in "Materials and Methods". The primer annealing sites are framed, the two for the β_2-adrenoceptor primers are shaded.

triuretic peptide (ANP), β_1-adrenoceptor, β_2-adrenoceptor, β_3-adrenoceptor and β-actin sequences. In addition, the 5'- and 3'-primer annealing sequences are separated by two single restriction sites (*BclI* and *XhoI*). These DNA-sequences (0.1 nmol of each) were treated in buffer containing 2 mmol/l $MgCl_2$/50 mmol/l KCl/10 mmol/l Tris-Cl, pH 8.3/0.2 µmol/l dNTPs/10 units *Taq* polymerase and incubated for 5 min at 95 °C (denaturation), 3 min at 64 °C (annealing) and 30 min at 72 °C for synthesizing the double-stranded DNA-sequence.

Using the outer primer-sequences (5'-atrial natriuretic peptide primer and 3'-β-actin primer) PCR was performed, producing an amplification product of 214 base pairs in length. The composition of the assay and the reaction conditions were as follows: 2 mmol/l $MgCl_2$/50 mmol/l KCl/10 mmol/l Tris-Cl, pH 8.3/0.2 µmol/l dNTPs/5 U *Taq* polymerase/2 ng double-stranded standard DNA and 50 pmol of 5'-atrial natriuretic peptide primer and 3'-β-actin primer. After a denaturation step at 95 °C for 5 min, 15 cycles of 95 °C/1 min, 50 °C/1 min, 72 °C/1 min were performed followed by 20 cycles of 95 °C/1 min, 40 °C/1 min, 72 °C/1 min with a finishing elongation step at 72 °C for 5 min. The resulting PCR-product (214 base pairs) was cloned into the single *SmaI* restriction site of the plasmid pGEM-4Z (Promega, Madison, WI, USA) containing the SP6/T7 RNA promoter sequence. RNA transcripts of this internal standard sequence were prepared as run-off transcripts by linearization at the *EcoRI* site located just 3' to the standard sequence. In vitro transcription was performed using a commercially available kit (Riboprobe Gemini System II; Promega, Madison, WI, USA). Subsequently the DNA-template was digested by adding 10 units DNAse I (FPLC*pure*, Pharmacia, Freiburg, Germany) to the reaction mixture and incubating for 20 min at 37 °C. After ethanol precipitation, the amount of internal standard RNA was determined at 260 nm. Absence of contaminating traces of remaining DNA template interfering in the competitive reverse transcription PCR assay was verified by subjection of the RNA to a PCR without reverse transcription obtaining no signal (negative control).

Competitive reverse transcription polymerase chain reaction

cDNA synthesis was carried out in three different 20 µl reverse transcription reactions each containing 200 ng of total RNA and various amounts of the corresponding internal standard RNA (0.3 pg, 3 pg, 30 pg). RNA was reverse transcribed in a buffer containing 5 mmol/l $MgCl_2$/50 mmol/l KCl/10 mmol/l Tris-Cl, pH 8.3/1 mmol/l dNTPs/2.5 µmol/l random hexamers/20 units of RNase inhibitor and 100 units of reverse transcriptase: The reaction was carried out at 42 °C for 20 min. Thereafter the mixture was incubated at 99 °C for 5 min to inactivate enzymatic activity. PCR was performed at final concentrations of 2 mmol/l $MgCl_2$/50 mmol/l KCl/10 mmol/l Tris-Cl, pH 8.3/0.2 mmol/l dNTPs/1.25 units of *Taq* polymerase and 12.5 pmol β_2-adrenoceptor primers in a total volume of 50 µl. Six µl of cDNA was added and PCR was performed with 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 62 °C for 1 min and chain extension at 72 °C for 1 min. All reverse transcription PCR additives were purchased from Perkin Elmer ("GeneAmp® RNA PCR KIT", Perkin Elmer, Überlingen, Germany).

Amplification products were 397 base pairs in length for the target RNA and 135 base pairs for the internal standard RNA.

High performance liquid chromatography

The products of the PCR reaction having different sizes were separated from nucleotides, primers and amplified primer dimers on a prepacked column with DEAE-bonded nonporous resin particles of 2.5 µm diameter (DEAE-NPR; no. N930-2656 from Perkin-Elmer). The HPLC device was a Waters LCM1 plus system consisting of two pumps 444, an automated gradient controller, an autosampler 712, a UV detector 486 and a data module 746 for registration and data analysis. Twenty to 80 µl from each PCR reaction (1 : 5 diluted with water) were injected into the column and the elution profile was registrated at 260 nm. The mobile phase consisted of various volume proportions of buffer A (25 mmol/l Tris-HCl, pH 9.0, plus 1 mol/l NaCl) and buffer B (25 mmol/l Tris-HCl, pH 9.0) in the following gradient program: 0.01 min, 46/54 (A/B); 0.50 min, 54/46; 8.00 min, 64/36; 10.00–12.00 min 46/54 to recalibrate the column. The total flow rate was 1 ml/min. The data module of the HPLC device translated the amount of PCR product into an area under the curve.

Quantification of β_2-adrenoceptor mRNA

Regression analysis of the integrated peak areas corresponding to the two specific PCR-products versus the amount of standard RNA used in a semi-logarithmic diagram, leads to the point of equivalence where the initial amount of target and standard RNA are the same (fig. 2). This point of equivalence represents the equimolar ratio of molecules of target and standard RNA initially present in the assay. Therefore the value obtained must be corrected for the different lengths of the PCR-fragments. For our β_2-adrenoceptor mRNA quantifications it has to be multiplied by the correction-factor of 135/397.

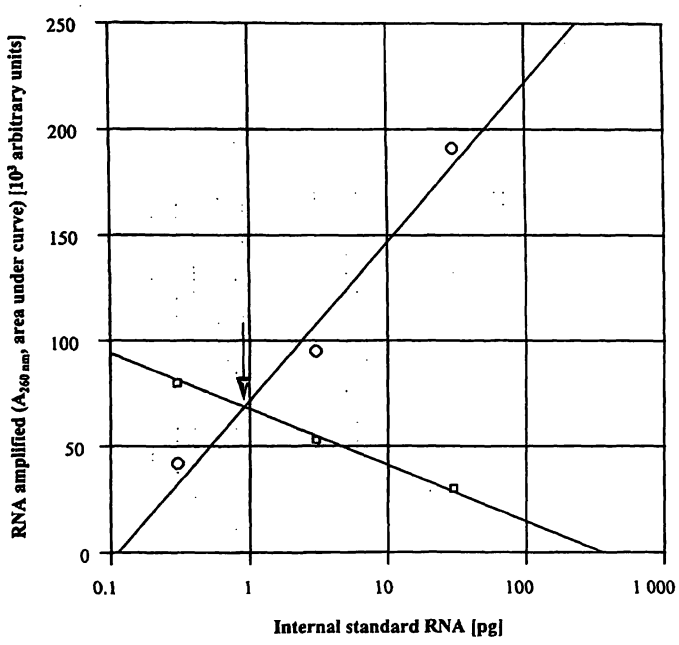

Fig. 2 Competitive reverse transcription PCR for quantification of β_2-adrenoceptor mRNA.
Two-hundred ng of total RNA was subjected to reverse transcription PCR together with 0.3 pg, 3 pg and 30 pg internal standard RNA, respectively. The resulting peak areas after HPLC-detection at 260 nm were plotted versus the amount of standard RNA used in the assay (semi-logarithmic). The intersection (arrow) shows the concentration of internal standard RNA used in the assay where equimolar amounts of the products arise.
□ β_2-adrenoceptor, o Internal standard.

Results

Exponential PCR amplification of β_2-adrenoceptor mRNA

To determine the exponential range of PCR amplification for β_2-adrenergic receptor mRNA and internal standard RNA, we coreverse-transcribed 400 ng of total cellular RNA isolated from heart tissue and 0.3 pg of internal standard RNA into the first-strand cDNAs. The cDNA products were then amplified for various numbers of cycles of PCR. PCR products were size-fractionated through anionic exchange HPLC, detected at 260 nm and the peak areas corresponding to the two PCR-products were integrated.

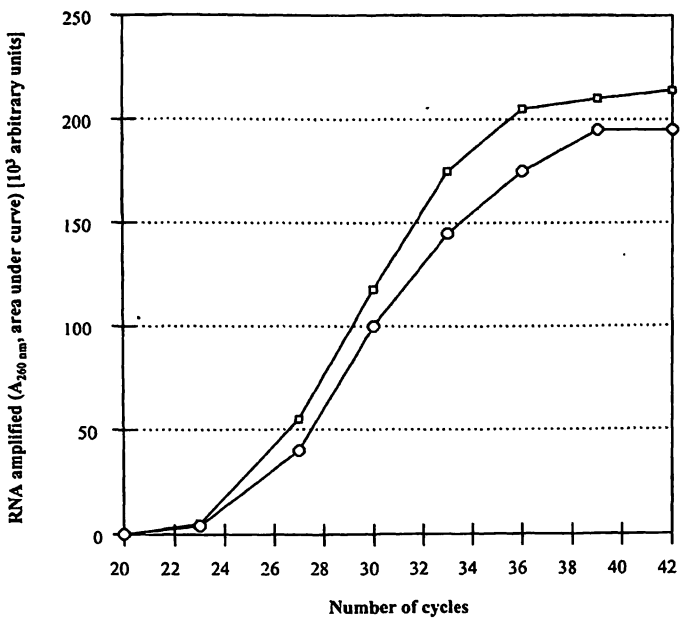

Fig. 3 Exponential range of amplification for 400 ng total cellular RNA and 0.3 pg of internal standard RNA.
Cellular and internal standard RNA were coreverse-transcribed. PCR of the cDNAs was performed for various numbers of cycles. The integrated peak areas are plotted on the y-axis.
□ β_2-adrenoceptor, o internal standard.

Figure 3 shows the results obtained from the exponential range experiment. Both the internal standard and the target cDNAs were amplified in an exponential fashion from 24 to 36 cycles. The relative amounts of the two amplified products stayed identical during the PCR amplification, even when the plateau phase was reached.

For exact quantification results in determination of patients' heart tissue RNA, we had to show that the internal standard and target cDNA are amplified with similar efficiencies in the PCR. Therefore 0.3 pg internal standard RNA and 400 ng total cellular RNA were reverse transcribed and subjected to 30 cycles of PCR (exponential range). Three different concentrations of the cDNA (6 µl concentrated, 6 µl 1 : 2, 6 µl 1 : 4) were used in the amplification reaction. Figure 4 shows that amplification rates of internal standard RNA and target RNA are almost the same. The parallelity of the lines demonstrates that the proportion of both cDNAs does not change during PCR.

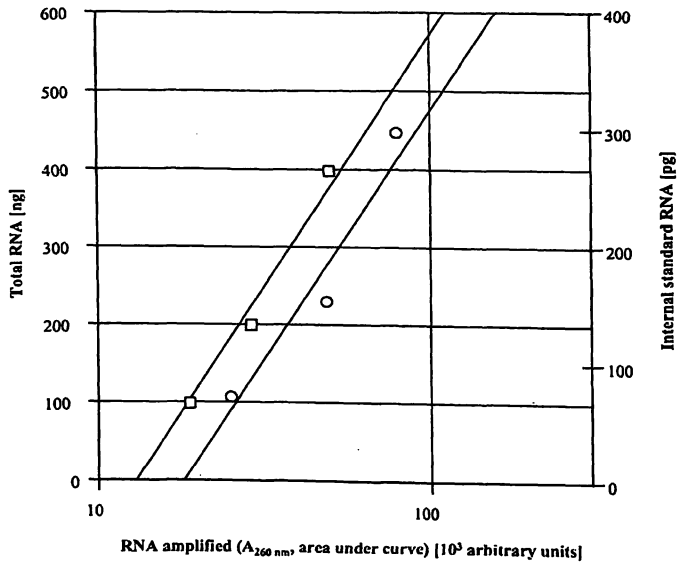

Fig. 4 Efficiencies of PCR-amplification for target- and standard-RNA.
Four-hundred ng total cellular RNA and 0.3 pg internal standard RNA were reverse transcribed and subjected to 30 cycles of PCR. Three different concentrations of the cDNA were used in the amplification reaction. The amplification rates are shown to be almost the same.
□ β_2-adrenoceptor, o internal standard.

HPLC analysis of PCR products

PCR products were separated from the various deoxynucleotides, primers, amplified primer-dimers and nonspecific products by employing an elution gradient optimized to separate DNA fragments in the range of 100 to 500 base pairs, that requires only 12 minutes cycle time per sample. The presence of two single peaks of PCR products with the expected size after HPLC analyses (after calibration with a DNA marker) demonstrated that none of our cDNAs had been contaminated with genomic DNA during the RNA extraction procedure.

Assay evaluation

Linearity

The following assay was performed to evaluate the linearity between the amount of initial cellular β_2-adrenoceptor mRNA and the PCR product obtained after 30

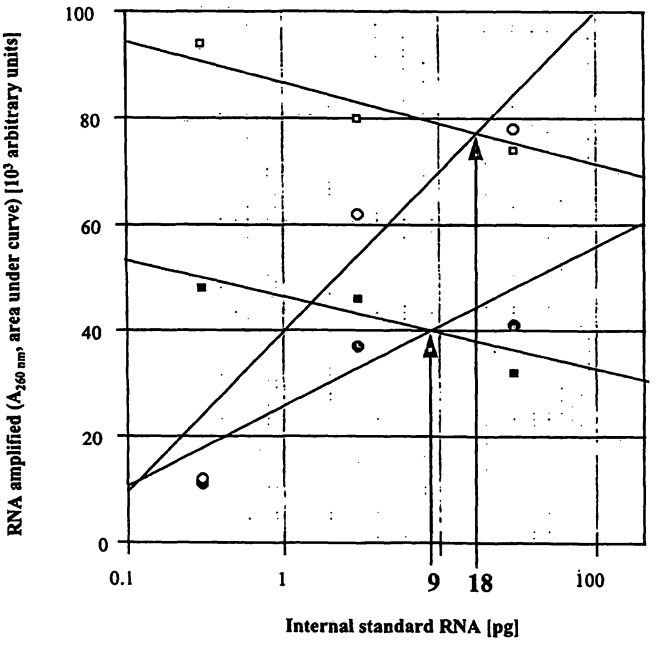

Fig. 5 Linearity of competitive reverse transcription PCR.
Two different amounts of the same cellular RNA (280 ng and 360 ng) were probed in a common competitive reverse transcription PCR assay for β_2-adrenoceptor mRNA (0.3 pg, 3 pg, 30 pg internal standard RNA). For 180 ng total cellular RNA we determined the intersection at 9 pg internal standard RNA and for 360 ng we found the point of equivalence at 18 pg internal standard RNA.
■ β_2-adrenoceptor (180 ng), ● internal standard (180 ng), □ β_2-adrenoceptor (360 ng), o internal standard (360 ng).

cycles of amplification. We probed two different amounts of the same cellular RNA in a common competitive reverse transcription PCR assay. Hundred and eighty ng and 360 ng of total RNA were both subjected to reverse transcription PCR, together with 0.3 pg, 3 pg and 30 pg β_2-adrenoceptor internal standard RNA. Evaluation of the data reveals a linear response. For 180 ng total cellular RNA, we determined the point of equivalence at 9 pg internal standard RNA and 360 ng total cellular RNA at 18 pg internal standard RNA (fig. 5).

Reliability of HPLC analysis

To evaluate internal variations of HPLC analysis, we subjected different amounts of the β_2-adrenoceptor PCR product to repeated HPLC analyses. In table 1, the area under the curve values of these experiments are shown. For a relatively constant standard deviation of about 2400, corresponding to the internal variation due to the baseline of the elution curve, the CV for the analysis clearly depends on the size of the area under the curve. Thus, to obtain an area under the curve with optimal reproducibility, or a low CV in this study, we used input cDNA volumes that would lead to PCR signals with an area under the curve > 30 000.

Tab. 1 Variation of HPLC analysis (area under the curve, arb. units) using PCR products of different concentrations.

Mean [arb. units]	SD [arb. units]	CV [%]	n
12 715	2328	18.3	9
24 876	2489	10.0	9
29 831	1950	6.5	9
43 214	2238	5.2	9
67 342	2631	3.9	9
93 418	2587	2.8	9

Reproducibility

We calculated the CV of competitive reverse transcription PCR by six replicate analyses of one sample (patient 2). The values varied from 12.7 to 19.3 $\times 10^6$ β_2-adrenoceptor molecules (12.7, 14.5, 15.8, 17.2, 18.0, 19.3) with a mean of 16.3 $\times 10^6$ β_2-adrenoceptor molecules and a CV of 15%. These variations take into account variations associated with cDNA synthesis, PCR amplification and HPLC analysis.

Accuracy and sensitivity

To test the accuracy of the method, we diluted total cellular RNA of one heart tissue preparation (patient 2, 16.3 $\times 10^6$ β_2-adrenoceptor molecules per µg total RNA) to get 6 different concentrations. The samples were subjected to reverse transcription PCR and analyzed as described. The experimental values plotted against the expected ones (fig. 6) showed the ability of competitive reverse transcription PCR to discriminate between different levels of β_2-adrenoceptor gene expression.

In repeated experiments, the sensitivity of the method was determined as 0.9 $\times 10^6$ β_2-adrenoceptor molecules per µg total RNA.

Quantification of β_2-adrenoceptor mRNA

For quantification of specific mRNAs, total cellular RNA of patient's heart tissues has to be isolated and

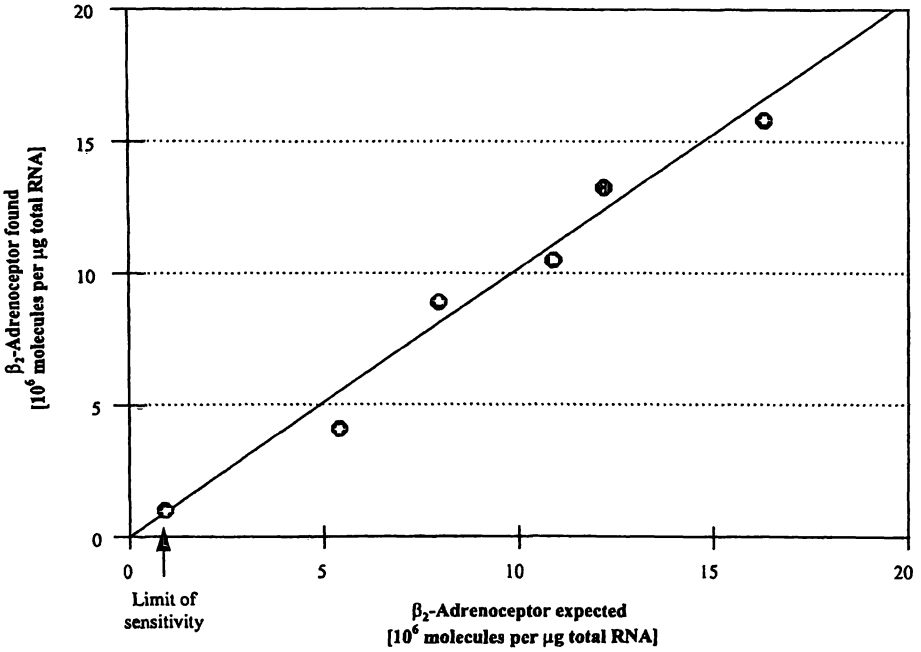

Fig. 6 Accuracy and sensitivity of competitive reverse transcription PCR. Total cellular RNA of one tissue preparation was diluted to 6 concentrations. Competitive reverse transcription PCR was performed, evaluated and the experimental values were plotted against the expected ones.

purified. Using the method of fractionated precipitation and anionic exchange chromatography we reached a yield of up to 50 µg RNA by preparation of approximately 170 mg human heart tissue. This in enough RNA for performing more than 50 quantification assays of β_2-adrenoceptor mRNA.

β_2-Adrenoceptor mRNA determination revealed that the mean value of the control group (15.6×10^6 β_2-adrenoceptor mRNA molecules per µg total RNA, tab. 2) is not statistically significantly different from the mean value of the group with β_1-blocker medication (22.7×10^6 β_2-adrenoceptor mRNA molecules per µg total RNA, *Mann-Withney*-test, $p < 0.5$). The mean values are in good agreement with data from *Ladenson* et al. (11) and *Bristow* et al. (8) investigating the endomyocardium and the left ventricular tissue, respectively. They give values of 15×10^6 determined in a patient with dilated cardiomyopathy and 22 mg $\pm$ 20×10^6 molecules β_2-adrenoceptor mRNA per µg total RNA in 12 patients with non-failing heart disease. Strikingly, our data show a high interindividual variability between the β_2-mRNA expression levels in the investigated patient groups also reported by *Bristow* et al. (8).

Tab. 2 β_2-Adrenoceptor mRNA quantification results of all patients included in the study.

Patient	Control group [10^6 molecules per µg RNA]	Patient	β_1-Antagonist therapy [10^6 molecules per µg RNA]
1	31.1	11	28.6
2	16.3	12	36.1
3	11.8	13	6.4
4	4.8	14	31.6
5	19.5	15	51.7
6	10.7	16	6.9
7	34.2	17	29.5
8	8.1	18	14.3
9	22.0	19	11.2
10	9.5	20	12.1
Mean	16.8	Mean	22.7
SD	9.9	SD	15.2

Discussion

Using the approach originally described by *Becker-André & Hahlbrock* (6), we have developed a new assay for the quantification of β_2-adrenoceptor mRNA expression by PCR. The synthetic β_2-adrenoceptor RNA, designed as an internal standard for this assay, controls the efficiency of both the reverse transcription reaction and the PCR. The synthetic RNA has the same primer sequences as the target mRNA, so that there are no differences in primer efficiencies. The difference in size between standard and target allows a simple and quick separation of the corresponding amplification products with anion-exchange HPLC.

Further, the constructed internal standard may also be used for quantification of atrial natriuretic peptide β_1, β_3- and actin mRNA, respectively. The results of PCR-based measurements of β_2-adrenoceptor mRNA in atrial tissues from patients with cardiac artery disease show a good correlation with studies in left ventricle myocardium tissues from patients with non-failing human heart (8).

The precision of the present non-radioactive assay is enhanced by the accurate measurement of the target and competitor mRNA using anion-exchange HPLC for separation and quantification. We obtained a dose-dependent linear response by using a constant amount of cellular RNA and increasing concentrations of competitor. These results demonstrate that the two cDNA species are equivalent in the relative efficiency of amplification. Imprecision for quantitative PCR assays with CV lower than 15% in this and other studies (12) were reported only for the PATTY procedure. Standardization against the housekeeping gene β-actin resulted in imprecision lower than 25% (13), whereas traditional methods of quantative PCR reported CVs as high as 40% (14, 15).

All the PCR experiments presented here were carried out with the same stocks of primers and internal standard mRNA. Indeed, we observed that PCR results could vary as much as 3-fold when we used different stocks of internal standard RNA, synthesized at a different time. Even higher variations with a 10-fold difference in amplification yields were reported when different stocks of primers were used (13). We speculate that the purity of the synthesized RNA might vary from lot to lot, although there was only one single band on the control gel electrophoresis.

HPLC has already been employed successfully for very precise PCR product quantification over a wide dynamic range of amplified DNA (9, 13, 16), possibly more precisely than it can be done by gel imaging or microplate-based techniques. While a single PCR product analysis is much faster by HPLC than by ethidium bromide-stained gel electrophoresis, multiple analysis may require more time, since only one sample can be assayed per run. As the resolution of the DEAE-NPR column permits linear gradients of only 12 minutes per run, this enables overnight analysis with the autosampler 712 of up to 48 samples.

In conclusion, the enhanced β_2-adrenoceptor sensitivity after β_1-blocker treatment remains unclear. The unchanged β_2-adrenoceptor mRNA expression in this study suggests that the relationship of steady state abundance of β_2-adrenoceptor mRNA and β_2-receptor protein levels in model systems (17, 18) may also exist in the human heart. The ability to measure steady-state

levels of cardiac mRNA provides a new approach to understanding the molecular and cellular mechanisms that contribute to myocardial disease.

Acknowledgements

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Corresponding author: Prof. Dr. Dr. H. Wisser, Department of Clinical Pathology, Robert-Bosch-Hospital, Auerbachstraße 110, D-70376 Stuttgart, Germany